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Different levels of Ras activity can specify distinct transcriptional and morphological consequences in early *Drosophila* embryos

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SUMMARY

The terminal portions of the *Drosophila* body pattern are specified by the localized activity of the receptor tyrosine kinase Torso (Tor) at each pole of the early embryo. Tor activity elicits the transcription of two 'gap' genes, *tailless* (*tll*) and *huckebein* (*hkb*), in overlapping but distinct domains by stimulating the Ras signal transduction pathway. Here, we show that quantitative variations in the level of Ras activity can specify qualitatively distinct transcriptional and morphological responses. Low levels of Ras activity at the posterior pole direct *tll* but not *hkb* transcription; higher levels drive transcription of both genes. Correspondingly, low levels of Ras activity specify a limited subset of posterior terminal structures, whereas higher levels specify a larger subset. However, we also show that the response to Ras activity is not uniform along the body. Instead, levels of Ras activity which suffice to drive *tll* and *hkb* transcription at the posterior pole fail to drive their expression in more central portions of the body, apparently due to repression by other gap gene products. We conclude that *tll* and *hkb* transcription, as well as the terminal structures, are specified by two inputs: a gradient of Ras activity which emanates from the pole, and the opposing influence of more centrally deployed gap genes which repress the response to Ras.

Key words: *Drosophila*, body pattern, gradients, Ras, terminalia

INTRODUCTION

Receptor tyrosine kinases are involved in transducing a variety of extracellular signals that elicit diverse cellular responses. Yet, transduction of these signals generally depends on a common intracellular pathway involving the small G-protein Ras, and the serine-threonine kinases Raf, MEK and MAPK (Marshall, 1994; McCormick, 1994; Marshall, 1995). Moreover, activity of this pathway alone is often sufficient to transduce the signal and lead to the expected cellular response (Perrimon, 1994; Kayne and Sternberg, 1995; Marshall, 1995). Thus, a central question is how activation of the Ras pathway can elicit so many distinct outputs, either in different cell types or through the exposure of equivalent cells to different kinds or amounts of ligand.

Specificity could be achieved in several ways. For example, cells of different type might express different constellations of transcription factors and hence be predisposed to respond to Ras activity in different ways (Brunner et al., 1994; Cowley et al., 1994). Alternatively, cells of the same type might express more than one receptor tryosine kinase and the activation of each of these receptors could produce a different output by engaging additional signal transduction pathways (Kazlauskas, 1994). Here, we consider a third way, namely that Ras activity may be continuously varied like a rheostat in response to different levels of a ligand and in this way provide a series of thresholds that elicit distinct outputs. Such a mechanism is of particular interest in developing tissues because some receptor tyrosine kinases are thought to mediate the patterned responses of cells to polarized or graded distributions of a given ligand (Casanova and Struhl, 1989; Sprenger and Nüsslein-Volhard, 1992; Casanova and Struhl, 1993; Katz et al., 1995; Schweitzer et al., 1995).

One instance of such a patterning phenomenon is the control of terminal body patterning in *Drosophila* embryos, which depends on the receptor tyrosine kinase Torso (Tor) (Schüpbach and Wieschaus, 1986; Nüsslein-Volhard et al., 1987; Klingler et al., 1988; Casanova and Struhl, 1989; Sprenger et al., 1989). Although Tor is expressed uniformly along the surface of the early embryo, it is activated only at the poles, apparently in response to the localized activity of an extracellular ligand, possibly the protein Trunk (Casanova and Struhl, 1989; Sprenger and Nüsslein-Volhard, 1992; Casanova and Struhl, 1993; Casanova et al., 1995). Activation of the Tor receptor then initiates a localized cascade of sequential Ras, Raf, MEK and MAPK activity within the embryo leading to the zygotic expression of two genes, *tailless* (*tll*) and *huckebein* (*hkb*), in overlapping but distinct domains at the poles (Pignoni et al., 1992; Lu et al., 1993a; Tsuda et al., 1993; Brunner et al., 1994; Casanova et al., 1994; reviewed by Lu et al., 1993b; Duffy and Perrimon, 1994). At least at the posterior pole, most if not all aspects of terminal patterning appear to be specified by the Tll and Hkb proteins which function as transcription factors to regulate the expression of a number of other target

genes (Pignoni et al., 1990; Weigel et al., 1990; Brönner and Jäckle, 1991). Experiments in which the level of Tor activity is controlled by temperature, rather than by the distribution of its ligand, have shown that high levels of Tor activity specify the more terminal structures (such as the anal pad and anal tuft at the posterior end), whereas lower levels specify less terminal structures (such as derivatives of abdominal segments A7 and A8; Casanova and Struhl, 1989). Thus, the localized activity of the Tor receptor might organize *tll* and *hkb* expression and thereby specify terminal body pattern by generating a Ras activity gradient (Casanova and Struhl, 1989; Sprenger et al., 1989; Furriols et al., 1996).

In this paper, we demonstrate that different levels of constitutive Ras activity can elicit distinct molecular outputs – the transcription of either *tll* alone, or *tll* and *hkb* together – as well as the formation of distinct portions of terminal body pattern. However, we also provide evidence that the response to a given level of Ras activity is not absolute but depends on cellular context, in particular on the presence or absence of other gap gene products. These findings indicate that cell pattern can be organized by mechanisms which depend on variations in both the level of Ras activity and in the responsiveness of cells to this activity.

MATERIALS AND METHODS

Composition of transgenes for ubiquitous expression of Ras^{V12} in early embryos

To generate early embryos in which the constitutively active Ras^{V12} protein is expressed ubiquitously, we constructed two transgenes in which the Ras^{V12} coding sequence was placed under the control of the promoter of either the *ribosomal protein 49* (*rp49*) gene (O'Connell and Rosbash, 1984; Kongsuwan et al., 1985) or the *Tubulinα1* (*Tubα1*) gene (Theurkauf et al., 1986). The *rp49* promoter fragment is an approx. 2 kb segment of DNA begining at a *Pst* site at the 5′ end and extending to the ATG at the 3′ end which has been mutated to GGTACC (a *Kpn*I site; D. Kalderon, personal communication). The *Tubα1* fragment has been described previously (Basler and Struhl, 1994). The *rp49* and *Tubα1* promoters direct low or moderate levels of transcription respectively, in most cells, as determined by examining the expression of *rp49-lacZ* and *Tubα1-lacZ* transgenes (G.S., unpublished findings). Because even low level constitutive expression of the Ras^{V12} coding sequence might be expected to be lethal, these transgenes were rendered conditional by inserting a Flp-out cassette between the promoter and coding sequence to terminate transcription (as in Struhl and Basler, 1993).

rp49>w⁺>ras^{V12} transgene

A genomic fragment containing the *rp49* promoter was placed upstream of the *ras^{V12}* coding sequence (Fortini et al., 1992) followed by the 3′ UTR of the *hsp70* gene (Struhl and Basler, 1993), and the resulting *rp49-ras^{V12}-hsp70 3′UTR* gene inserted into a derivative of the *Carnegie 20* transformation vector (Rubin and Spradling, 1982) lacking the ry^+ rescuing marker. A $>w^+>$ Flp-out cassette was then constructed by introducing the w^+ minigene derived from the *Casper* transformation vector (Pirrotta, 1988) between two minimal FRTs in the J33 plasmid (Struhl and Basler, 1993) and the cassette inserted between the *rp49* and *ras^{V12}* sequences to create the final *rp49>w⁺>ras^{V12}* transgene.

Tubα1>w⁺>ras^{V12}

A similar strategy was followed to that described for the *rp49>w⁺>ras^{V12}* transgene except that a genomic fragment containing the *Tubα1* promoter (Basler and Struhl, 1994) was used in place of the *rp49* promoter fragment.

Genetics

Generating *1X* and *2X rp49>ras^{V12}* embryos

A balanced stock of the following genotype was generated by standard crosses: *y w; tor^{RX} hsp70-flp.2/CyO; rp49>w⁺>ras^{V12}/TM2*. Late larvae and early pupae from this stock were heat shocked at 37°C for 60 minutes to excise the $>w^+>$Flp-out cassette from most of the resident transgenes (Struhl and Basler, 1993) and adult *tor^{RX}/tor^{RX}* females carrying either one or two copies of the transgene were selected based on the presence or absence of the *TM2* balancer chromosome. These females were crossed to wild-type males and allowed to lay eggs for 3-4 days before embryos were collected for analysis (to ensure that the mutant embryos derive exclusively from mutant female germ cells, rather than from mosaic nurse cell/oocyte complexes).

Generating *1X rp49>ras^{V12} bcd osk tsl* embryos

Females of the genotype *y w hsp70-flp.1; bcd^{E1} osk^{166} tsl^{691}/TM2* were crossed to males of the genotype *y w; rp49>w⁺>ras^{V12}; bcd^{E1} osk^{166} tsl^{691}* to generate *y w/y w hsp70-flp.1; rp49>w⁺>ras^{V12}/+; bcd^{E1} osk^{166} tsl^{691}/ bcd^{E1} osk^{166} tsl^{691}* females which were treated as described above. To obtain *bcd osk* or *bcd osk tsl* females carrying a single copy of the *Tubα1>rasV12* transgene, *y w hsp70-flp.1; bcd^{E1} osk^{166} tsl^{691}, Tubα1>w⁺>ras^{V12}/TM2* females were crossed to either *bcd^{E1} osk^{166}* or *bcd^{E1} osk^{166} tsl^{691}* males.

In situ analysis

RNA in situs were performed as described by Jiang et al. (1991) with the following modifications. For double labelling experiments, one RNA probe was labelled with dioxigenenin-conjugated UTP while the other probe was labelled with flourescein-conjugated UTP. Both probes were hybridized at the same time and then detected sequentially using blue and red alkaline phosphate substrate reactions (as described by Strahle et al., 1994). For single labelling experiments, probes were labelled with dioxigenenin-conjugated UTP. Plasmids containing *tll* (Pignoni et al., 1990) and *hkb* (Brönner and Jäckle, 1991) cDNAs were a gift from J. Casanova (Casanova et al., 1994). Genomic PCR using the primers CGGAATTCGATCGAACATCCAGGG and CGGCGCTAAGCTATTCC was performed to obtain a 483 bp DNA fragment from the 3′ region of the *byn* transcript (Kispert et al., 1994; Singer et al., 1996). This fragment was subcloned into bluescript (Stratagene) and used for the production of RNA probes.

Cuticular analysis

Embryos were allowed to develop for 24 hours and then mounted in a 1:1 mixture of Hoyers mountant and lactic acid (Struhl, 1984).

RESULTS

Molecular and morphological responses to localized Tor activity at the posterior pole

The Tor receptor, as well as Ras, Raf, MEK and MAPK, are required for the normal patterns of transcription of the gap genes *tll* and *hkb* at each end of the early embryo. The products of these gap genes then, in turn, specify the pattern of the most anterior and most posterior portions of the body (see Introduction; Fig. 1). At the posterior pole, the requirement for Tor and the Ras/Raf signal transduction pathway is absolute – neither *tll* nor *hkb* are expressed in their absence, and no terminal structures form. In contrast, both *tll* and *hkb* are expressed at the anterior pole in the absence of terminal

signaling, albeit in abbreviated domains, under the control of the anterior determinant Bicoid (Pignoni et al., 1992; Liaw and Lengyel, 1993). In the present study, we are concerned with how localized Tor activity organizes different patterns of gene expression and cuticular differentiation; hence, to simplify the analysis, we focus on these responses in the posterior half of the body.

As shown previously (Pignoni et al., 1990; Brönner and Jäckle, 1991) and in Fig. 1, *hkb* and *tll* are expressed in overlapping domains which extend approximately 8% and 15% egg's length (EL) from the posterior pole in syncytial blastoderm embryos (late stage 4 and early stage 5; staging as in Campos-Ortega and Hartenstein (1985)). The combined activities of *tll* and *hkb* then specify the more complex patterns of expression of subordinate transcription factors such as *forkhead*, *hunchback*, *AbdominalB* (*AbdB*), *cad*, and *brachyenteron* (*byn*) (Casanova, 1990; Weigel et al., 1990; Kispert et al., 1994; Singer et al., 1996; reviewed by Jürgens and Hartenstein, 1993). For example, *tll* activates *byn* transcription, while *hkb* represses it, resulting in a stripe of *byn* expression (Kispert et al., 1994; see Fig. 1). All of these gene functions appear to play significant roles in directing the formation of the posterior terminalia, including the ventral dentical belt of the eighth abdominal segment (A8), the posterior spiracles, the anal pad and tuft, and internal structures such as the hindgut, posterior midgut, and Malpighian tubules (Casanova, 1990; Jürgens and Hartenstein, 1993).

Different levels of Ras activity specify distinct transcriptional responses.

To test whether different levels of Ras activity can suffice to specify distinct transcriptional responses at the posterior pole, we sought to generate embryos that lack Tor, but express different levels of a constitutively active form of Ras, Ras^{V12} (Trahey and McCormick, 1987). This was accomplished by creating females with the following genetic properties. First, they were homozygous for a null allele of the *tor* gene: embryos developing from such mutant females lack Tor-dependent activity of endogenous Ras. Second, they carried one or two copies of a Flp-out transgene, *rp49>w$^+$>ras^{V12}*, composed of the promoter from the ubiquitously expressed *ribosomal protein 49* (*rp49*) gene (O'Connell and Rosbash, 1984), a *w$^+$* Flp-out cassette which blocks transcription (*>w$^+$>*), and the *ras^{V12}* coding sequence (Materials and Methods). Finally, they carried an *hsp70-flp* transgene in which the coding sequence for the yeast recombinase Flp is placed under the control of the *Drosophila hsp70* heat shock promoter. Late third instar larvae and early pupae of this genotype were heat shocked to catalyze the excision of the Flp-out cassette from most of the resident *rp49>w$^+$>rasV12* transgenes (see Materials and Methods). We refer to embryos derived from such *tor*$^-$ female germ cells as *1X rp49>ras^{V12}* and *2X rp49>ras^{V12}* embryos depending on whether the females carried one or two copies of the transgene. By the same convention, we refer to embryos derived simply from *tor*$^-$ or wild-type females as *0X rp49>ras^{V12}* or *ras$^+$* respectively.

As shown in Fig. 1, neither *tll* nor *hkb* transcripts are expressed posteriorly in *0X rp49>ras^{V12}* embryos. However *tll* is expressed in a narrow domain at the posterior of *1X rp49>ras^{V12}* embryos and in a broader domain in *2X rp49>ras^{V12}* embryos. We also found that *tll* appears to be expressed at higher level at the posterior pole of *2X rp49>ras^{V12}* embryos compared to the posterior pole of *1X rp49>ras^{V12}* embryos (data not shown). In contrast, we failed to detect posterior *hkb* expression in *1X rp49>ras^{V12}* embryos, but could readily detect *hkb* transcription at the posterior pole of *2X rp49>ras^{V12}* embryos. *hkb* is expressed at the posterior pole of these *2X rp49>ras^{V12}* embryos in a domain somewhat narrower, and at a level somewhat lower, than that seen in *ras$^+$* embryos.

We draw three conclusions from these results. First, the different levels of constitutive Ras^{V12} activity in *1X* and *2X rp49>ras^{V12}* embryos appear to distinguish between two qualitatively distinct transcriptional outputs: *tll* alone, and *tll* plus *hkb*. Second, the level of Ras^{V12} activity is also related quantitatively to target gene transcription, as the higher level of Ras^{V12} activity in *2X rp49>ras^{V12}* embryos appears to generate a higher level of posterior *tll* expression than that generated in *1X rp49>ras^{V12}* embryos. Third, even though we would anticipate that Ras^{V12} is active uniformly throughout these embryos, we find that *tll* and *hkb* are only transcribed in tightly restricted domains at the poles, with the boundary of the *tll* domain depending on the different levels of Ras^{V12} activity generated in *1X* and *2X rp49>ras^{V12}* embryos. These last results suggest the existence of a local differential in the ability of nuclei to respond to a constant level of Ras^{V12} activity.

We also examined the transcription of the *byn* gene, in *1X* and *2X rp49>ras^{V12}* embryos (Fig. 1). In *1X rp49>ras^{V12}* embryos we observe that *byn* transcripts are expressed in a small cap (approx. 9% EL; egg length), whereas the domain of expression in *2X rp49>ras^{V12}* embryos is significantly broader (approx. 15% EL). We note that *byn* expression extends back to the posterior end of *2X rp49>ras^{V12}* embryos, unlike in *ras$^+$* embryos in which *byn* expression is normally repressed at the posterior pole. Because both the activation and repression of terminal *byn* expression are known to depend, respectively, on *tll* and *hkb*, we surmise that higher levels of Ras activity are required at the posterior of wild-type (*ras$^+$*) embryos to drive sufficiently high levels of Hkb expression to repress *byn* expression. These results reinforce those obtained for *tll* and *hkb* transcription and further suggest that both these genes are regulated quantitatively as well as qualitatively by the level of Ras activity.

Different levels of Ras activity specify distinct terminal structures

We next examined the effects of different levels of Ras activity on the differentiation of terminal structures. As shown in Fig. 1, *1X rp49>ras^{V12}* embryos show a modest restoration of posterior terminal structures which are absent in the *0X rp49>ras^{V12}* embryos. In particular, these embryos form the least terminal of the posterior terminal structures: the eighth abdominal dentical band and the posterior spiracles. The extent of restoration is considerably greater in *2X rp49>ras^{V12}* embryos: these form additional terminal structures such as the anal tuft and anal pads. As observed for the patterns of *tll* and *hkb* transcription in *2X rp49>ras^{V12}* embryos, these structures appear in the normal spatial order, and hence the external cuticular pattern of these embryos is similar to that of wild-type (*ras$^+$*) embryos. Thus, we conclude that different levels of Ras can elicit the formation of distinct subsets of terminal

pattern elements, just as they distinguish between different transcriptional outputs.

Different responses to a constant level of Ras activity

As noted above, the localized expression of *tll* and *hkb* at the posterior poles of *1X* and *2X rp49>ras^{V12}* embryos indicates that nuclei located at different positions along the anteroposterior axis of late syncytial embryos do not respond in the same way to a constant level of Ras^{V12}. The gap genes *hunchback* (*hb*), *Krüppel* (*Kr*), *knirps* (*kni*), and *giant* (*gt*) are expressed in overlapping central domains of the body, organized in large part by the anterior and posterior determinants Bicoid (Bcd) and Oskar (Osk) (reviewed by St. Johnston and Nüsslein-Volhard, 1992). All four of these gap genes encode transcription factors, and at least some are capable of acting as repressors (reviewed by Gray and Levine, 1996). Hence, it is possible that the localized expression of one or more of these factors blocks the response of nuclei in more central portions of the body to Ras activity.

To test this possibility, we have examined the consequences of generating uniform Ras^{V12} activity in embryos lacking the anterior and posterior determinant systems as well as Tor receptor activity. This was accomplished by creating females that (i) were homozygous for recessive loss-of-function mutations of the *bcd*, *osk*, and *torso-like* (*tsl*) genes (the *tsl* gene is required during oogenesis for the activity of the Tor receptor during embryogenesis; Stevens et al., 1990; Savant-Bhonsale and Montell, 1993; Martin et al., 1994), (ii) carried the *hsp70-flp* transgene and (iii) carried one copy of either the *rp49>w$^+$>ras^{V12}* transgene or a *Tubα1>w$^+$>ras^{V12}* transgene which has a stronger ubiquitous promoter derived from the *Tubulinα1* (*Tubα1*) gene (Materials and Methods).

In the absence of exogenous Ras^{V12} activity, embryos derived from *bcd$^-$ osk$^-$ tsl$^-$* females express *Kr* uniformly and at high level, but lack detectable *hb*, *kni*, *gt*, *tll* and *hkb* transcription (Struhl et al., 1992; data not shown). These embryos also express moderate levels of Hb protein derived from ubiquitous, maternally derived *hb* transcripts. When *bcd$^-$ osk$^-$ tsl$^-$* females carrying a single copy of either the *rp49>w+>ras^{V12}* or *Tubα1>w$^+$>ras^{V12}* transgene were heat shocked as late larvae or early pupae, they gave rise to embryos that expressed *tll* uniformly throughout the body but failed to express *hkb* (Fig. 2B and data not shown). Thus, all of the nuclei in these embryos appear to respond similarly to low to moderate levels of constitutive Ras^{V12} activity by transcribing *tll*, but not *hkb*.

As shown in Fig. 1G, doubling the doseage of the *rp49>ras^{V12}* transgene in *tor$^-$* females generates embryos that have sufficient Ras^{V12} activity to direct *hkb* expression at the posterior pole, and a similar result is obtained using a single copy of the *Tubα1>ras^{V12}* transgene in place of two copies of the *rp49>ras^{V12}* transgene (Fig. 2A). Nevertheless, the same level of constitutive Ras^{V12} activity is not sufficient to drive

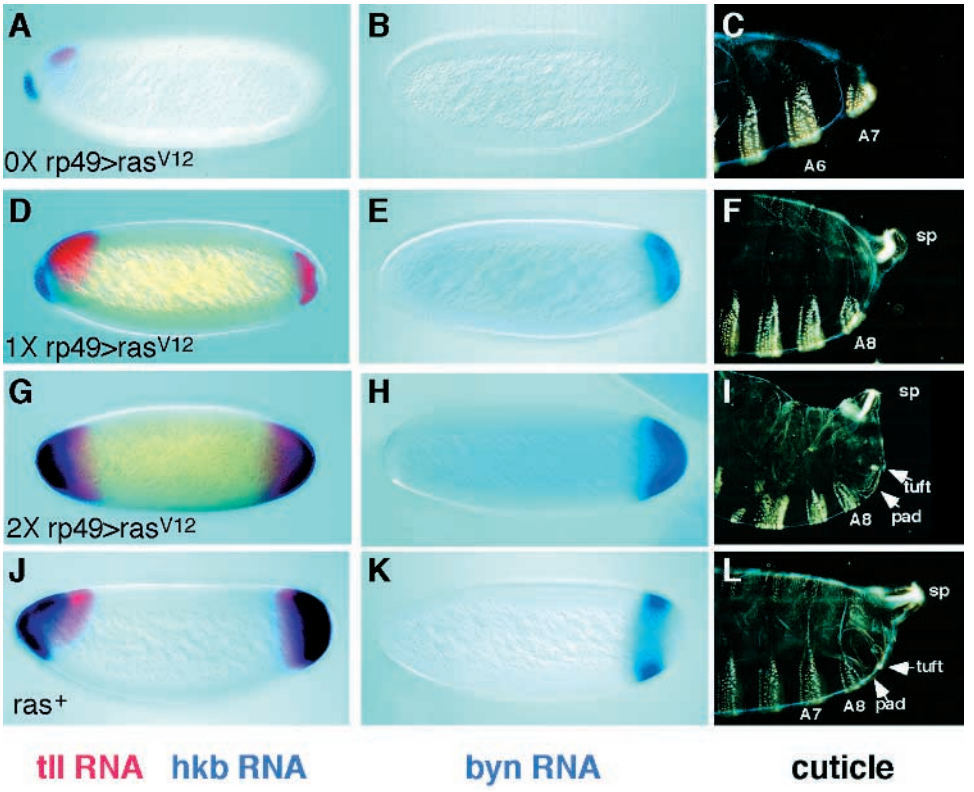

Fig. 1. Qualitatively distinct responses in embryos containing different levels of Ras activity. Embryos in which Ras activity derives solely from 0 (A-C), 1 (D-F), or 2 (G-I) copies of the *rp49>ras^{V12}* transgene or from the wild-type *ras$^+$* gene (J-L) are shown stained for *tll* (red) and *hkb* (blue) expression in A,D,G and J, and for *byn* expression in B,E,H and K; the posterior terminal structures formed by these embryos are shown in C,E,I and L (see text for detailed description of genotypes). In wild-type embryos (*ras$^+$*), *tll* and *hkb* are expressed in overlapping domains at the posterior pole (J) and activation by *tll* and repression by *hkb* results in a stripe of *byn* expression (K). *tll* and *hkb* also direct the expression of other downstream genes whose combined activities specify the posterior terminalia (L) such as the eighth abdominal denticle belt (A8), the anal pad and tuft, the posterior spiracles (sp), as well as internal structures, such as the hindgut. In *0X rp49>ras^{V12}* embryos, *tll*, *hkb* and *byn* are not expressed at the posterior pole (A,B) and no posterior terminal structures are formed (C). In contrast, the low level of Ras activity present in *1X rp49>ras^{V12}* embryos is sufficient to induce *tll* and *byn* expression, but not *hkb* expression, in a narrow domain at the pole (D,E) and to specify the formation of those terminal structures, such as the A8 ventral denticle belt and the spiracles, that normally arise farthest from the posterior pole (F). Both *tll* and *hkb* are expressed in *2X rp49>ras^{V12}* embryos (G), with *tll* expressed in both a broader domain and higher levels than in *1X rp49>ras^{V12}* embryos (D). The domain of *byn* expression also expands and appears to be expressed at higher levels (H). Finally, the levels of Ras activity in *2X rp49>ras^{V12}* embryos are sufficient to specify most or all of the exterior terminal structures, including the anal pad and anal tuft (I).

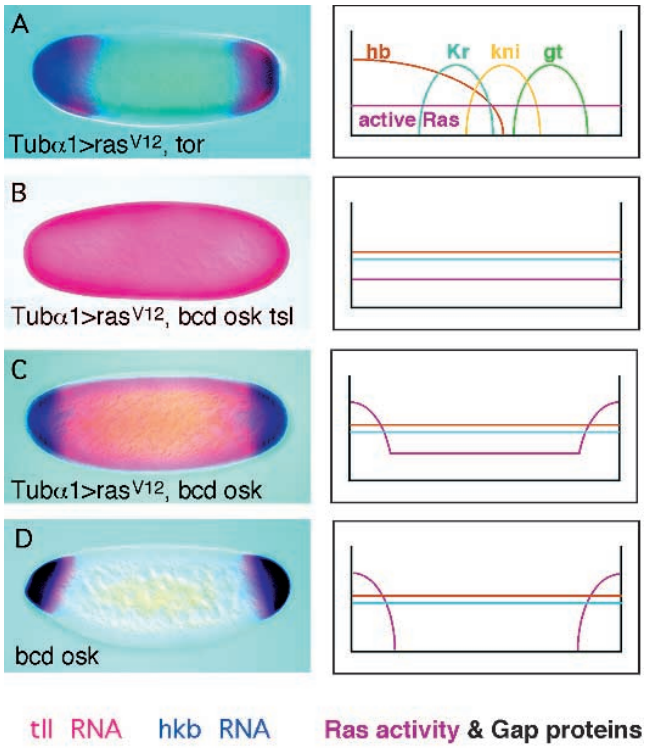

Fig. 2. Opposing roles of Ras and centrally expressed gap genes in controlling *tll* and *hkb* transcription. The diagrams adjacent to each embryo represent, in simplified form, early gap gene expression and Ras activity in the embryo. In embryos derived from *tor*$^{-}$ females carrying one copy of the *Tubα1>ras*^{V12} transgene (A), as in embryos derived from *tor*$^{-}$ females carrying two copies of the *rp49>ras*^{V12} transgene (Fig. 1G-I), moderate levels of uniform Ras^{V12} activity induce *tll* and *hkb* expression at the poles. In embryos derived from *bcd*$^{-}$ *osk*$^{-}$ *tsl*$^{-}$ females (B), Hb and Kr are expressed uniformly and Kni and Gt expression are repressed by Hb (Struhl et al., 1992); under these conditions, moderate levels of uniform Ras^{V12} activity derived from the *Tubα1>ras*^{V12} transgene drive *tll*, but not *hkb*, transcription throughout the embryo. *hkb* expression is, however, restored at the posterior pole of embryos derived from *bcd*$^{-}$ *osk*$^{-}$ females carrying one copy of the wild-type *tsl* allele and one copy of the *Tubα1>ras*^{V12} transgene (C), presumably due to high levels of endogenous Ras activity generated by the Tor receptor. As shown in (D), this endogenous Ras activity is sufficient to specify essentially normal domains of expression of both *tll* and *hkb* in embryos derived from *bcd*$^{-}$ *osk*$^{-}$ females that lack Ras^{V12} activity, even though the remaining gap gene products are initially either absent or uniformly expressed.

hkb transcription in embryos lacking all three determinant systems, even at the posterior pole (Fig. 2B). We note that the particular constellation of gap gene proteins uniformly expressed in embryos from *bcd*$^{-}$ *osk*$^{-}$ *tsl*$^{-}$ females (High Kr, moderate Hb, no Kni and no Gt; Struhl et al., 1992) is not found at the posterior pole of embryos derived from either wild-type or *tor*$^{-}$ females. Thus, the abnormal presence of Hb and/or Kr throughout these embryos might be responsible for their failure to express *hkb* in response to moderate Ras^{V12} activity.

To ask whether a higher level of Ras activity can elicit *hkb* transcription in embryos lacking both the anterior and posterior determinant systems, we examined *tll* and *hkb* transcription in embryos derived from *bcd*$^{-}$ *osk*$^{-}$ females carrying a single copy of the *Tubα1>ras*^{V12} transgene. These embryos retain wild-type *tsl* function and hence give rise to embryos in which ubiquitous Ras^{V12} activity is supplemented at the poles by normal levels of endogenous Ras activity. As shown in Fig. 2C, they showed localized transcription of *hkb* at both poles, presumably in response to normal, localized activity of endogenous Ras. Thus, we infer that higher levels of Ras activity generated under these conditions can suffice to drive *hkb* transcription.

Finally, it is informative to compare the patterns of *tll* and *hkb* transcription at the posterior poles of embryos derived from *bcd*$^{-}$ *osk*$^{-}$ females (Fig. 2D) with those derived from *tor*$^{-}$ females which carry one copy of the *Tubα1>ras*^{V12} transgene (Fig. 2A). In the former, these patterns depend solely on the normal, localized activity of the Tor receptor, and hence on the polarized activity of wild-type Ras. In the latter, the only Ras activity is that of the uniformly expressed Ras^{V12} protein; consequently, the restricted expression of *tll* and *hkb* presumably reflects the ability of the remaining gap gene products to repress Ras-dependent transcription of *tll* and *hkb*. Nevertheless, each input can generate a correctly ordered pattern of *tll* and *hkb* expression, suggesting that they function in a cooperative fashion to organize *tll* and *hkb* expression during normal development.

DISCUSSION

During early embryogenesis, terminal patterning is organized by a mechanism involving local activation of a transmembrane receptor tyrosine kinase, Tor, and transduction by the Ras signal transduction pathway (reviewed by Perrimon, 1993). Here, we show that different levels of a constitutively activated form of Ras, Ras^{V12}, are able to generate different transcriptional responses and specify the formation of distinct structures at the posterior end of the body. Low levels of Ras^{V12} suffice only to direct *tll* transcription and the formation of posterior structures such as the A8 ventral denticle band and the posterior spiracles, whereas higher levels induce both *tll* and *hkb* transcription and specify additional, more terminal structures such as the anal pads and anal tuft. Thus, our results establish that quantitative variation in the level of the Ras activity can lead to qualitatively distinct outcomes, and hence suggest that localized activition of the Tor receptor organizes terminal body pattern by creating a gradient of activity of the Ras signal transduction pathway. Our results also draw attention to the presence of another significant influence on terminal body pattern, namely the localized deployment of other gap gene products in more central portions of the body. At least one of these gap gene products appears to dampen or block the responsiveness to activated Ras, counteracting the influence of the Ras activity gradient emanating from the pole.

Generation and interpretation of a Ras activity gradient

The receptor Tor is thought to be activated by an extracellular ligand that is generated locally at each pole and then adsorbed and sequestered by binding to the receptor (Stevens et al., 1990; Sprenger and Nüsslein-Volhard, 1992; Casanova and Struhl, 1993). Diffusion of the ligand before binding might suffice to generate a graded distribution of activated Tor and hence of activated Ras. It is also possible that formation of the

Ras activity gradient depends at least in part on subsequent movement of activated Tor, Ras or other downstream signaling components within the syncytial embryo.

The most likely intracellular targets of the Ras transduction pathway are factors which regulate *tll* and *hkb* transcription. In the case of *tll*, analysis of *cis*-acting regulatory sequences upstream of the promoter suggests that activity of the Ras pathway normally activates *tll* transcription by antagonizing the action of a ubiquitously expressed transcriptional repressor which binds to these sequences (Liaw et al., 1995). One candidate for this repressor is the product of *grainyhead* gene, a homologue of the vertebrate *NFT-1* gene. *grainyhead* function is apparently required to prevent general transcription of the endogenous *tll* gene (Liaw et al., 1995). Moreover, MAPK is capable of phosphorylating NTF-1 in vitro (Liaw et al., 1995). Hence, a Ras activity gradient might generate a graded distribution of phosphorylated Grainyhead protein, relieving repression of *tll* transcription when a sufficient fraction of the protein is phosphorylated and hence inactivated.

A similar mechanism could also apply to the localized transcription of *hkb*. For example, if Grainyhead were to act directly as a repressor of both *tll* and *hkb*, different levels of the active form of the protein might suffice to repress each gene, and hence, different levels of Ras activity would be required to release them from repression. Alternatively, other transcription factors that have a different sensitivity to the Ras signal transduction pathway may be responsible for regulating *hkb* transcription.

Whatever the mechanism, our findings indicate that the *tll* and *hkb* genes respond in qualitatively distinct ways to different levels of Ras activity and suggest that a relatively small difference in Ras activity – that resulting from a two-fold increase in the maternal dose of the *rp49>ras^{V12}* transgene – can suffice to distinguish between the 'off' and 'on' states of transcription. Thus, the threshold sensitivity of *tll* and *hkb* to differences in Ras activity may be similar to that exhibited by other gap genes in response to the Bicoid and Hunchback gradient morphogens (Struhl et al., 1989, 1992).

Although most, or all, aspects of posterior terminal pattern are governed by the actions of Tll and Hkb protein, the resulting patterns of gene expression and cuticular differentiation are complex, involving overlapping patterns of transcription of many target genes such as *byn*, *fkh*, *caudal* and *abdB*, and the formation of diverse morphological structures. Previous studies (Strecker et al., 1988; Casanova, 1990; Diaz et al., 1996) have provided evidence that this complexity arises in part from the formation of local gradients of Tll and Hkb expression within the domains in which the two genes are transcribed. We have observed that the levels of both *tll* and *hkb* transcription appear to be sensitive to the level of Ras activity. For example, *tll* is expressed at higher level at the posterior end of *2X rp49>ras^{V12}* embryos compared to *1X rp49>ras^{V12}* embryos, and the level of posterior *hkb* transcription appears similarly dependent when 2X *rp49>rasV12* and *ras^{+}* embryos are compared. As illustrated by the transcription of *byn* in these embryos, quantitative differences in *tll* and *hkb* transcription appear to correlate with corresponding differences in the transcription of further downstream target genes. Thus, the activity gradient of Ras may be translated into local gradients of *tll* and *hkb* which in turn have instructive roles in organizing subordinate gene expression and differentiation.

A complicating factor in interpreting the organizing influence of the Ras activity gradient is the role played by other gap genes, which appear to modulate the response to Ras activity. Our results establish that nuclei at different positions along the anteroposterior axis are predisposed to respond in distinct ways to the same level of constitutive Ras^{V12} activity. Moreover, they indicate that this predisposition results from the localized activities of one or more of the remaining gap genes in more central portions of the body. In the posterior half of the body, the gap proteins Hunchback (Hb), Krüppel (Kr), Knirps (Kni) and Giant (Gt) are expressed in an ordered series of overlapping domains, and these are organized principally in response to the anterior and posterior determinants Bicoid (Bcd) and Osk (Osk). Hence, irrespective of the activity of the terminal determinant system, nuclei at different positions from the posterior pole will be exposed to different constellations of these proteins, all of which are DNA binding transcription factors. As shown in Figure 2, localized expression of these 'central' gap gene proteins can suffice to organize overlapping posterior domains of *tll* and *hkb* transcription in *1X Tubα1>ras^{V12}* embryos in which all nuclei are exposed to the same level of Ras activity.

Thus, nuclei in the posterior half of the body are subject to two, independent inputs which control the expression of *tll* and *hkb*: a Ras activity gradient spreading from the posterior pole which drives their expression, and an opposing gradient of one or more central gap gene proteins which repress their expression. Under the appropriate circumstances, either input can generate a correctly ordered pattern of *tll* and *hkb* transcription. In the wild-type condition, the two inputs may function in a mutually reinforcing fashion to generate the normal patterns of transcription of both target genes. This situation is analogous to the opposing roles of Bcd and Osk in organizing the expression of the central gap genes themselves (Hulskamp et al., 1989; Irish et al., 1989; Struhl, 1989; Wharton and Struhl, 1991; Struhl et al., 1992), and may reflect a common strategy used in pattern formation. As is the case for Bcd and Osk, the opposing activities of the Ras gradient and the central gap genes also appear to be mutually exclusive. Conditions which force high levels of ectopic gap gene expression at the posterior pole block terminal development, probably through the repression of *tll* and *hkb* transcription (Gaul and Jäckle, 1989; Struhl, 1989a). Conversely, conditions which force high levels of ectopic Tor or Ras activity in the central portion of the body can block transcription of the central gap genes (Klingler et al., 1988; Casanova and Struhl, 1989; Steingrimsson et al., 1991). A similar phenomenon of mutual exclusion and collaboration between opposing signals is observed in the developing adult legs, where Wg and Dpp restrict each other's expression defining the dorso-ventral axis, but operate in conjunction to establish the proximo-distal axis (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996).

A rheostat function for Ras: implications for other systems

As noted in the Introduction, receptor tyrosine kinases are capable of transducing a variety of signals through the Ras pathway, in some cases generating distinct outputs to different ligands or to different concentrations of the same ligand (reviewed by Marshall, 1995). Our present demonstration that

different levels of a constitutively active form of Ras can elicit distinct transcriptional outputs indicates one mechanism by which this specificity can be achieved. In essence, Ras can function as a rheostat in which quantitative variations in activity provide one or more thresholds that differentially regulate gene expression. This mechanism may be particularly important in developmental contexts in which the graded or localized distribution of ligand appears to organize cell pattern. In addition to terminal body patterning, receptor tyrosine kinases have been implicated in a number of patterning phenomena of this kind in *Drosophila*, including organization of the follicular epithelium during oogenesis and control of dorso-ventral epidermal pattern in the embryo (Clifford and Schüpbach, 1989; Price et al., 1989; Brand and Perrimon, 1994; Schweitzer et al., 1995). A rheostat mechanism might also be involved in situations in which cells of the same type can respond in distinct ways to different ligands, each received by a different receptor tyrosine kinase (Marshall, 1995). A possible example of this phenomenon in *Drosophila* is the R7 cell in the *Drosophila* eye, which requires the activity of both Sevenless and the EGF receptor during development (Campos-Ortega et al., 1979; Tomlinson and Ready, 1987; Xu and Rubin, 1993; Freeman, 1996).

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